XP-000891783

MOLECULAR AND CELLULAR BIOLOGY, Nov. 1993, p. 7029–7035
0270-7306/93/117029-07$02.00/0

P.D. 11-1993
p. 7029-7035 = 7

Vol. 13, No. 11

Identification of Distinct Roles for Separate E1A Domains in Disruption of E2F Complexes

MASA-AKI IKEDA AND JOSEPH R. NEVINS*

Section of Genetics, Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

Received 12 May 1993/Returned for modification 2 July 1993/Accepted 24 August 1993

The adenovirus E1A protein can disrupt protein complexes containing the E2F transcription factor in association with cellular regulatory proteins such as the retinoblastoma gene product (Rb) and the Rb-related p107 protein. Previous experiments have shown that the CR1 and CR2 domains of E1A are required for this activity. We now demonstrate that the CR2 domain is essential for allowing E1A to interact with the E2F-Rb or the E2F-p107-cyclin A-cdk2 complex. Multimeric complexes containing E1A can be detected when the CR1 domain has been rendered inactive by mutation. In addition, the E1A CR1 domain, but not the CR2 domain, is sufficient to prevent the interaction of E2F with Rb or p107. On the basis of these results, we suggest a model whereby the CR2 domain brings E1A to the E2F complexes and then, upon a normal equilibrium dissociation of Rb or p107 from E2F, the E1A CR1 domain is able to block the site of interaction on Rb or p107, thereby preventing the re-formation of the complexes.

A variety of experiments over the past 2 years have documented the role of the E2F transcription factor as a target for the Rb protein as well as other cell cycle regulatory proteins (35). The interaction of Rb with E2F, which inhibits the transcriptional activating capacity of E2F (7, 17, 19, 45, 50), coincides with the ability of Rb to function as a growth suppressor (38, 39). E2F is also found in association with the Rb-related p107 protein. The interaction of p107 with E2F appears to allow the formation of a larger, multimeric complex involving the cyclin A polypeptide and the cdk2 protein kinase (4, 9, 43). This complex forms at the beginning of S phase (34), consistent with the kinetics of appearance of cyclin A (37). Recent experiments also demonstrate the presence of additional E2F-p107 complexes in G_1, including one that contains the cyclin E protein together with the cdk2 kinase (28).

Both in vivo and in vitro assays have demonstrated the ability of the adenovirus E1A protein to disrupt these E2F complexes, resulting in the release of uncomplexed, transcriptionally active E2F (1, 2, 5, 6, 34). This activity is dependent on E1A sequences found within conserved domains 1 and 2 (CR1 and CR2) (40), sequences also known to be important for the oncogenic activity of E1A and the binding of E1A to cellular proteins such as Rb and p107 (21, 29, 33, 46, 47, 51). These are also sequences that are shared in the T-antigen gene and the E7 gene of simian virus 40 (SV40) and human papillomavirus, respectively (14). Indeed, it is also now clear that both T antigen and E7 share the ability to disrupt E2F complexes (5). Although E2F is utilized by the adenovirus E2 promoter, E2F is not a factor used for either SV40 transcription or human papillomavirus transcription (27). Rather, the importance of E2F for these viruses, as well as for adenovirus, would appear to lie in the fact that E2F is involved in the activation of a group of S-phase genes such as DHFR (3, 32, 44). Very likely, the activation of E2F by these viral oncoproteins facilitates the entry into S phase, creating a suitable environment for the replication of viral DNA.

Although the ability of these viral proteins to disrupt the E2F complexes is now well documented, the actual mechanism for this dissociation has not been addressed. Previous experiments have indicated that while both CR1 and CR2 of E1A are required for dissociation of E2F complexes and the CR1 domain was sufficient for E1A to block formation of the complexes, the CR2 domain was not required for E1A to block formation of the complexes (20, 40). This result, together with the observation that CR1 and CR2 appear to bind independently to the Rb protein (11), suggested the possibility of distinct roles for these E1A domains in the dissociation event. We have now sought to extend these studies to elucidate the process whereby E1A mediates the release of E2F from interactions with Rb as well as p107.

MATERIALS AND METHODS

Cells and extract preparation. U937 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Nuclear extracts were prepared as described previously (10).

In vitro translation of E1A proteins and isolation of glutathione S-transferase (GST) fusion proteins. The pGEM1 plasmids containing the wild-type $E1A_{12S}$, the $E1A_{pm928}$ mutant, and the $E1A_{\Delta 38\text{-}67}$ mutant have been described previously (40). The cDNAs were transcribed and translated in vitro with the TNT reticulocyte lysate transcription and translation kit from Promega Biotechnology. The construction, expression, and purification of GST-E1A fusion proteins ($E1A_{12S}$, $E1A_{pm928/961}$, and $E1A_{\Delta 38\text{-}67}$), GST-Rb fusion proteins, and GST-p107 fusion proteins have been described elsewhere (13, 23, 26). The wild-type GST-Rb fusion protein contains Rb residues from 373 to 792. A mutant Rb fusion protein, derived from an exon 22 Rb deletion, is missing amino acid residues 738 to 775. The wild-type GST-p107 fusion protein contains p107 residues 252 to 816 as described in Ewen et al. (13). A mutant p107 protein contains a phenylalanine substitution for cysteine 713.

Peptide synthesis. The following E1A peptides were used in this study: HFEPPTLHELYDLDVTA (CR1, residues 37 to 53), DLTCHEAGFPPSDDEDEEG (CR2, residues 121 to

* Corresponding author.

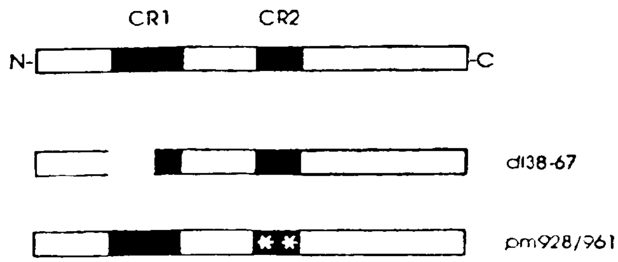


FIG. 1. Schematic depiction of the E1A functional domains and mutants utilized for dissociation assays. The wild-type 243-amino-acid product of the $E1A_{12S}$ cDNA is depicted at the top. The two regions of conserved sequence (CR1 and CR2) (25) have been shown to be important for binding to Rb and p107 proteins (47) and for dissociation of E2F-containing complexes (1, 40). The deletion in the $E1A_{dl38-67}$ mutant, which involves amino acid residues 38 to 67, is depicted by a gap. The positions of point mutations in the $E1A_{pm928/961}$ mutant are indicated by asterisks, and the name of the mutant indicates the nucleotide positions of the mutations.

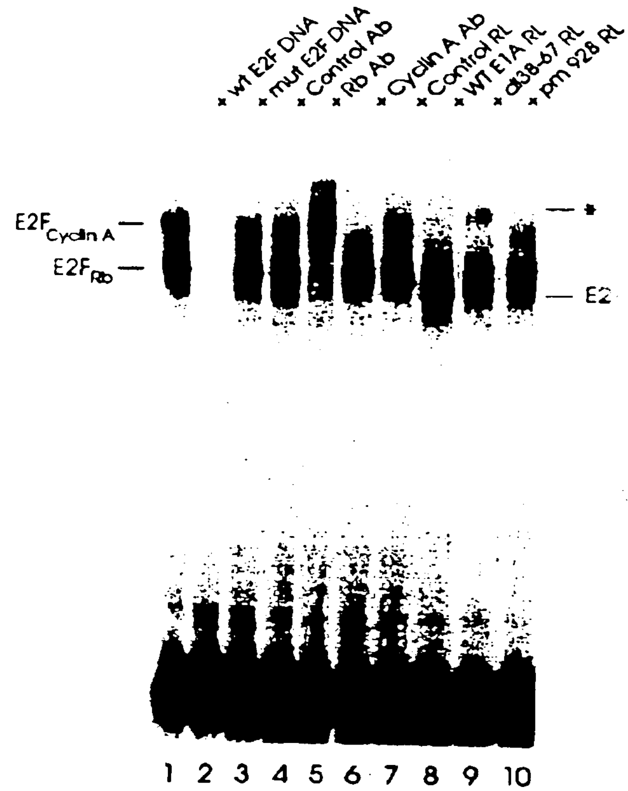


FIG. 2. The CR1 and CR2 domains of E1A are required for E2F complex dissociation. A U937 cell extract (2 μg) was added to the DNA binding reaction mixture with a control reticulocyte lysate (lanes 1 to 7) or equal amounts (1 μl) of a reticulocyte lysate programmed with the wild-type $E1A_{12S}$ cDNA (lane 8), the $E1A_{dl38-67}$ mutant (lane 9), or the $E1A_{pm928}$ mutant (lane 10). At the same time, 1 μl of the following solutions was added: 20 ng of cold E2F wild-type competitor DNA (lane 2), 20 ng of cold E2F mutant competitor DNA (lane 3), a c-*fos* monoclonal antibody (2G9C3) (lane 4), an Rb monoclonal antibody (C36) (lane 5), the cyclin A antiserum (lane 6), and normal rabbit serum (lane 7). The reaction mixes were preincubated for 20 min on ice and then incubated for another 20 min at room temperature. Conditions for DNA binding and gel electrophoresis are described in Materials and Methods. The positions of the E2F-cyclin A complex, the E2F-Rb complex, and free E2F are indicated. The reduced mobility complex resulting from the addition of the $E1A_{dl38-67}$ mutant protein is indicated by the asterisk.

139), HFEPPTLHELYDLDVTAPDLTCHEAGFPPSDDEDEEG (CR1-CR2, residues 37 to 54 and 121 to 139), HFKPPTDHKHTDDDVTAPDLTCHEAGPPPSDDEDEEG (CR1 mutant CR1-CR2), and HPEPPTLHELYDLDVTAPDKTGHKAGFPPSLGKDKKG (CR2 mutant CR1-CR2).

Antibodies. The Rb monoclonal antibody C36 and the c-*fos* monoclonal antibody 2G9C3 were obtained from Oncogene Science. The E1A monoclonal antibody M58, the Rb monoclonal antibody XZ91, the cyclin A monoclonal antibody BF683, and the c-*myc* monoclonal antibody 6E10 were obtained from PharMingen. The cyclin A antiserum has been described previously (36).

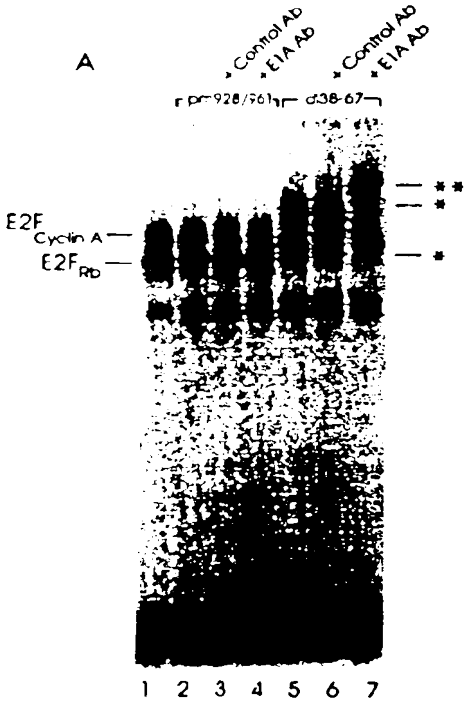
E2F gel shift assay. E2F DNA binding assays contained 2 μg of nuclear extract in 10 μl of DNA binding reaction mixture {20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.9], 40 mM KCl, 6 mM $MgCl_2$, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid], 1 mM dithiothreitol, 0.1% Nonidet P-40, 10% glycerol, 30 μg of bovine serum albumin, 500 ng of sonicated salmon sperm DNA} and 0.1 ng of ^{32}P-labelled DNA probe. The DNA probe is an *Eco*RI-*Hin*dIII fragment from the ATF(−) adenovirus E2 promoter plasmid (30). The sequences of double-stranded oligonucleotides used as cold competitors in E2F DNA binding assays were as follows: $E2F_{WT}$, TCC GTT TTC GCG CTT AAA TTT GAG AAA GGG CGC GAA ACT GGA, and $E2F_{mut}$, TCC GTT GTC GAG CTT AAA TTT GAG AAA GGG CTC GAC ACT GGA. In addition, either 0.5 to 1 μl of 1:1 diluted reticulocyte lysate or 0.5 μl of casein (10 mg/ml) (Hammerstein grade) was added to each reaction mixture to eliminate nonspecific protein-DNA interaction. The reaction mixtures were incubated for 20 min at room temperature and resolved in a 4% polyacrylamide gel in TBE (50 mM Tris-borate, 1 mM EGTA) for 3 h at 300 V at 4°C. For some experiments, 5% glycerol was added to the gel to improve the resolution of E2F complexes.

Competition assay of E2F-Rb or E2F-p107 complexes with E1A peptides. Partially purified E2F was prepared from HeLa cell whole-cell extracts through a fast-performance liquid chromatography Mono Q column as described previously (19). One microliter of partially purified E2F was incubated with the GST-Rb fusion protein (100 ng) or the GST p107 fusion protein (100 ng) in the presence of various concentrations (20 ng to 2 μg) of an E1A CR1 or CR2 peptide in the DNA binding reaction mixture containing 0.5 μg of casein per μl on ice for 20 min. The reaction mixture was incubated for an additional 20 min at room temperature and resolved in a 4% polyacrylamide gel containing 5% glycerol.

RESULTS

Stable interaction of an E1A CR1 mutant with E2F complexes. Previous experiments have demonstrated the importance of the CR1 and CR2 domains of E1A in the dissociation of E2F from complexes containing Rb or p107-cyclin A-cdk2 (1, 2, 6, 34, 40). To further address the specific roles of these sequences, we have made use of E1A mutants that

A



B

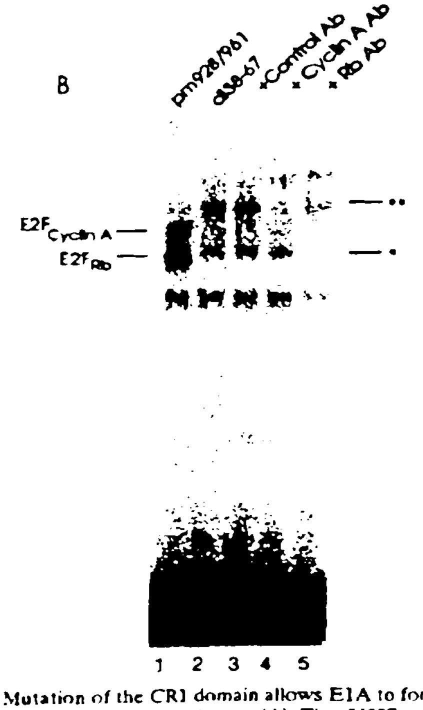


FIG. 3. Mutation of the CR1 domain allows E1A to form a stable interaction with the E2F complexes. (A) The U937 extract was preincubated in a DNA binding reaction mixture with equal amounts (500 ng) of the GST-E1A$_{pm928/961}$ protein (lanes 2 to 4) or the GST-E1A$_{dl38-67}$ protein (lanes 5 to 7). Incubation was for 20 min on ice and then continued with equal amounts (500 ng) of a control *c-myc* monoclonal antibody (6E10) (lanes 3 and 6) or an E1A monoclonal antibody (M58) (lanes 4 and 7) for another 20 min at room temperature. E2F binding reactions were carried out under the same conditions as described for Fig. 2 and resolved in a 4% polyacrylamide gel containing 5% glycerol. Complexes of reduced mobility resulting from the addition of the E1A mutant (•) as well as the E1A antibody (••) are indicated. (B) The U937 extract was preincubated in a DNA binding reaction mixture with equal amounts (500 ng) of the GST-E1A$_{pm928/961}$ protein (lane 1) or the GST-E1A$_{dl38-67}$ protein (lanes 2 to 5). Incubation was for 20 min on ice and then continued with equal amounts (200 ng) of a control *c-myc* monoclonal antibody (6E10) (lane 3), a cyclin A monoclonal antibody (BF683) (lane 4), or a Rb monoclonal antibody (XZ91) (lane 5) for another 20 min at room temperature. Complexes of reduced mobility resulting from the addition of the E1A mutant that contain the Rb protein (•) or the cyclin A protein (••) are indicated.

alter the function of these domains. The E1A$_{dl38-67}$ mutant deletes essential sequences from CR1, whereas the E1A$_{pm928/961}$ mutant contains a double point mutation within CR2 (Fig. 1). In some experiments, we have made use of a mutant with a single point mutation in CR2, E1A$_{pm928}$, which exhibits a phenotype identical to that of the double point mutant. The effect of these mutations on the ability of E1A to disrupt E2F complexes is shown in Fig. 2. Extracts of the human cell line U937 contain both the E2F-Rb complex and the E2F-p107-cyclin A-cdk2 complex (hereafter referred to as the E2F-cyclin A complex) as demonstrated by the recognition of Rb and cyclin A with specific antibodies (lanes 5 and 6). Whereas the wild-type E1A protein was able to disrupt each complex, resulting in the release of free E2F (lane 8), addition of either the E1A$_{dl38-67}$ mutant (lane 9) or the E1A$_{pm928}$ mutant (lane 10) failed to dissociate the E2F complexes.

Although neither E1A mutant was able to dissociate the E2F complexes, it was evident that the CR1 mutant did have an effect on the E2F-DNA complexes. Whereas the addition of the E1A$_{pm928}$ CR2 mutant did not alter the pattern (Fig. 2, lane 10), addition of the E1A$_{dl38-67}$ CR1 mutant resulted in an apparent shift of the complexes to a slower mobility. This was particularly evident with the E2F-cyclin A complex (Fig. 2, lane 9). The effect of the E1A$_{dl38-67}$ CR1 mutant on the E2F-Rb complex is more apparent in the experiment whose results are shown in Fig. 3A, utilizing a modification of the electrophoresis system that improves separation of the complexes. It is clear from this assay that the addition of a GST-E1A$_{dl38-67}$ mutant protein to the U937 cell extract resulted in a reduction in the mobility of both the E2F-cyclin A complex and the E2F-Rb complex (lane 5). In contrast, addition of the *pm*928/961 mutant had no effect (lane 2). Confirmation of the identity of the E1A-shifted complexes as containing cyclin A and Rb was provided by the addition of monoclonal antibodies specific to these proteins (Fig. 3B).

These results suggested that the E1A CR1 mutant protein was able to interact with each of these complexes and thus retard the mobility. To provide direct evidence for such an interaction, we have made use of an E1A monoclonal antibody. As seen in Fig. 3A (lane 7), the addition of the E1A antibody further reduced the mobility of each of these complexes, thus demonstrating the presence of the mutant E1A protein in the complexes. From these results, we conclude that whereas an E1A protein that is defective in the CR2 domain (*pm*928/961) produces no alteration in the pattern of E2F complexes, an E1A protein with a defective CR1 domain (*dl*38-67) has the capacity to form a stable interaction with the E2F-Rb complex as well as the E2F-p107-cyclin A-cdk2 complex.

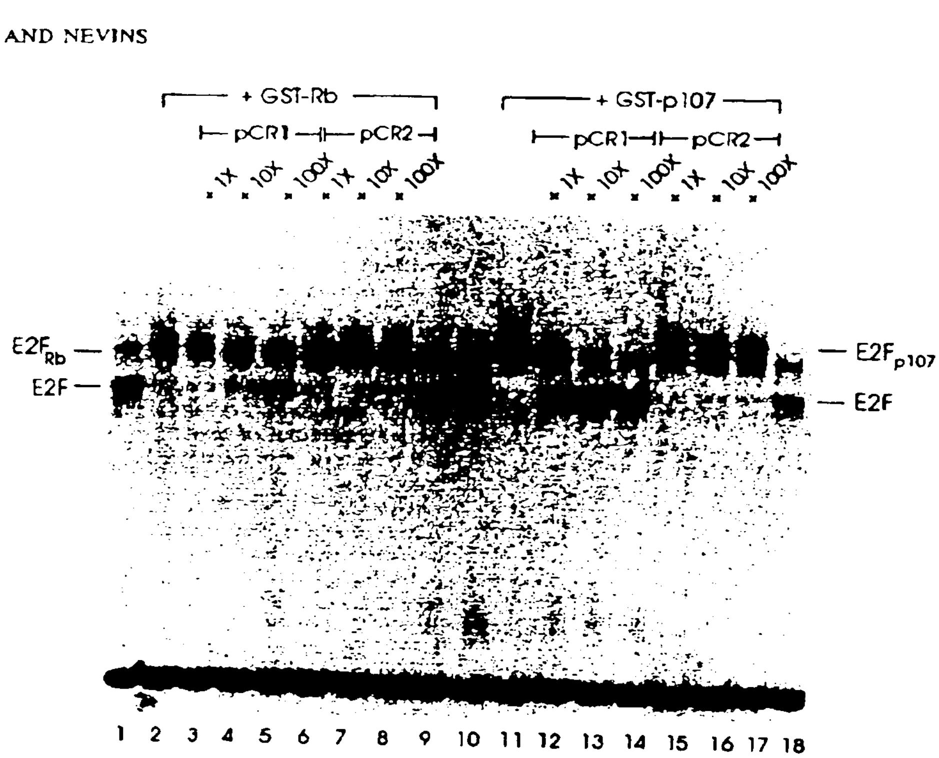


FIG. 4. Inhibition of E2F-Rb and E2F-p107 complex formation by an E1A-CR1 peptide. A partially purified preparation of E2F was incubated with 100 ng of the GST-Rb fusion protein or the GST-p107 fusion protein in the presence or absence of various concentrations (20 ng = 1×) of E1A CR1 (pCR1) or CR2 (pCR2) peptides. The addition of a GST-Rb mutant and a GST-p107 mutant is shown in lanes 10 and 18, respectively. Incubation was for 20 min on ice in the DNA binding mix and then for an additional 20 min at room temperature. E2F binding was assayed as described in Materials and Methods. Assay of E2F alone is shown in lane 1.

Role of E1A CR1 and CR2 in E2F complex dissociation. Our previous experiments have shown that the E1A CR1 domain was capable of blocking the formation of an E2F complex, whereas both CR1 and CR2 were essential for dissociation of the complex (40). The data presented in Fig. 3 demonstrate that an E1A protein with a defective CR1 domain can form a stable complex with the E2F-Rb or E2F-cyclin A complexes. Since the E1A$_{pm928/961}$ CR2 mutant is defective for dissociation of either of the E2F complexes and the CR1 domain appears to be sufficient to block complex formation (40), it would appear that the role of the CR2 domain may be to bring E1A to the E2F complex to then allow the CR1 domain to block an interaction.

We have addressed the respective roles of the CR1 and CR2 domains by measuring the ability of synthetic peptides representing CR1 and CR2 sequences to block the interactions in a variety of assays. First, we have assessed the ability of these peptides to block formation of the E2F complexes involving either the Rb protein or the p107 protein. As shown in Fig. 4, it is possible to reconstitute an E2F-Rb complex (lane 2) and an E2F-p107 complex (lane 11) with partially purified E2F and GST fusion proteins. Although the addition of the CR2 peptide had no effect on the formation of the complexes, addition of the CR1 peptide blocked the formation of each complex although there were clearly apparent differences in the efficiency. For instance, whereas the CR1 peptide blocked formation of the E2F-p107 complex at the lowest level tested (20 ng), inhibition of E2F-Rb complex formation required at least a 100-fold higher concentration of peptide. This quantitative difference is consistent with other findings indicating a higher affinity of the CR1 sequence for p107 than for Rb (11). Thus, consistent with previous assays with E1A mutants, it appears that the CR1 region is sufficient to block formation of the E2F complexes.

A second assay measured the ability of the E1A peptides to interfere with the capacity of wild-type E1A protein to disrupt the E2F complexes. If the first step in the E1A-mediated dissociation involves the CR2-mediated interaction of E1A with the E2F-Rb or E2F-p107 complex, then we would anticipate that a CR2 peptide might compete with the wild-type E1A protein for these interactions and thus prevent complex dissociation. As shown in Fig. 5, addition of the wild-type E1A protein to a U937 extract resulted in the disruption of the two prominent E2F complexes (lane 4). Addition of the CR1 peptide had no effect on this dissociation (lane 5), but addition of the CR2 peptide blocked the ability of wild-type E1A to disrupt the complexes (lane 6). Thus, consistent with the analysis of E1A mutants, the CR2 domain is essential for the dissociation event.

Finally, using these E1A peptides, we have addressed the role of the E1A sequences in allowing formation of the multimeric complexes containing the E1A CR1 mutant, E2F, and the associated proteins. As shown in Fig. 6, addition of the E1A$_{dl38-67}$ mutant again resulted in the formation of the multimeric complexes with reduced mobility (lane 4). Whereas the CR1 peptide did not inhibit the formation of these complexes (lane 5), addition of the CR2 peptide abolished the ability of the CR1 mutant to interact with either the E2F-Rb or the E2F-cyclin A complex (lane 6). It did appear that the addition of the CR1 peptide, in the presence of the E1A$_{dl38-67}$ mutant, resulted in some dissoci-

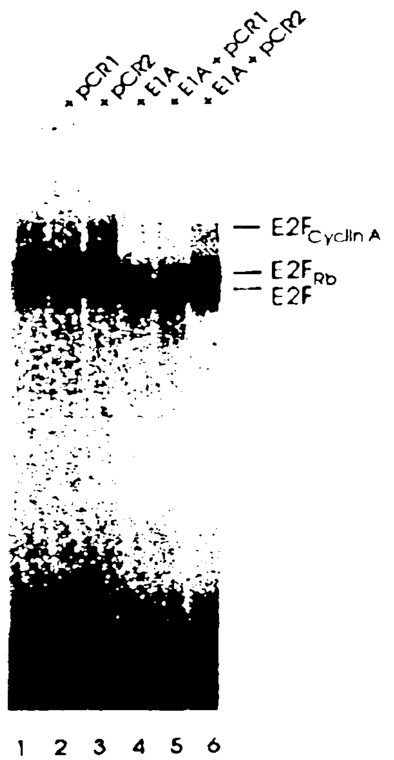


FIG. 5. The E1A CR2 peptide blocks the E1A-mediated dissociation of E2F complexes. The U937 cell extract was preincubated in DNA binding reaction mixture with 300 ng of CR2-mutated CR1-CR2 peptide or CR1-mutated CR1-CR2 peptide for 20 min on ice, and then equal amounts (1 μl) of a control nonprogrammed reticulocyte lysate (lanes 1 to 3) or a reticulocyte lysate programmed with the wild-type $E1A_{12S}$ cDNA (lanes 4 to 6) were added to the reaction mixtures. The incubation was continued for an additional 20 min at room temperature. E2F binding was assayed by gel retardation as described in the legend to Fig. 2. Mobility of the E2F-Rb complex, the E2F-cyclin A complex, and free E2F is as indicated.

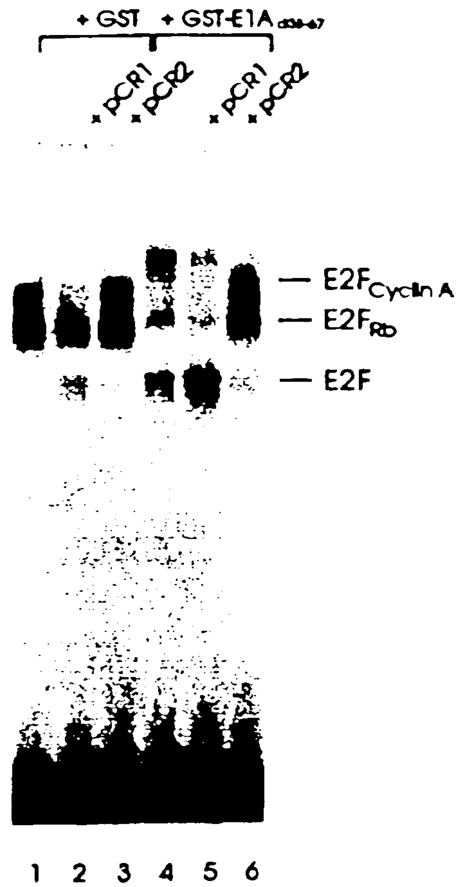


FIG. 6. The E1A CR2 peptide blocks the formation of E1A-containing complexes. The U937 cell extract was preincubated in DNA binding reaction mixture with 900 ng of CR2 mutated CR1-CR2 peptide or CR1 mutated CR1-CR2 peptide for 20 min on ice, and then equal amounts (500 ng) of a control GST protein (lanes 1 to 3) or the GST-$E1A_{dl30-67}$ protein (lanes 4 to 6) were added to the reaction mixtures. The incubation was continued for 20 min on ice and then for an additional 20 min at room temperature. E2F binding was assayed by gel retardation as described in the legend to Fig. 3. Mobility of the E2F-Rb complex, the E2F-cyclin A complex, and free E2F is as indicated.

ation of the E2F complexes and release of free E2F. Possibly, the interaction of the E1A mutant protein with the E2F complex results in a destabilization such that a high concentration of the CR1 peptide can cause limited dissociation. Regardless, the clear conclusion from these assays is that the interaction of E1A with the E2F complexes is dependent on the E1A CR2 domain.

DISCUSSION

Previous experiments have documented the ability of the adenovirus E1A protein, as well as the SV40 T antigen and the human papillomavirus E7 product to disrupt complexes containing the E2F transcription factor (1, 2, 5, 6, 34). The experiments we report here suggest a mechanism by which this dissociation might occur, at least for the action of the adenovirus E1A protein. Specifically, our assays demonstrate distinct roles for the two functional domains of E1A that are required for the dissociation event. As shown in Fig. 7, we consider the possibility that the E2F-Rb complex (the same arguments would also hold for the E2F-cyclin A complex) is in equilibrium with the dissociated components, in this case E2F and Rb. The extent of the equilibrium within the cell is not clear, although under most normal circumstances one can detect very little free E2F. This equilibrium is also likely influenced by the phosphorylation state of the Rb protein since it would appear that only the underphosphorylated form of Rb can interact with E2F (6, 41).

Our previous experiments have shown that the CR1 domain of E1A is sufficient to block the formation of an in vitro-reconstituted E2F complex (40) that is now known to represent the E2F-p107-cyclin A-cdk2 complex. Our experiments have extended this observation by using the purified p107 protein as well as the Rb protein to reconstitute the complexes. In each case, the CR1 sequence, in the form of a synthetic peptide containing only the CR1 domain, was able to inhibit complex formation, although it was clear that inhibition of formation of the E2F-Rb complex was less

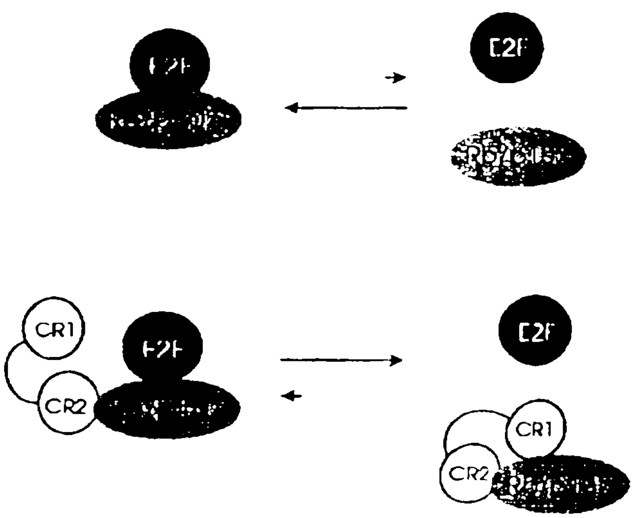


FIG. 7. A model for the E1A-mediated dissociation of the E2F complexes.

efficient than the inhibition of formation of the E2F-p107 complex. The experiments we describe here now demonstrate that the CR2 domain very likely allows the initial interaction of E1A with the E2F complex. As indicated in Fig. 7, we suggest that the two domains of E1A recognize distinct regions of the Rb protein. On the basis of all of these data, we suggest that the role of E1A CR2 domain is to bring the E1A protein to the complex. Then, as dissociation occurs in the normal equilibrium conditions, the CR1 domain would interact with the Rb sequences involved in the E2F recognition and thus prevent re-formation of the complex. By so doing, E1A would drive the equilibrium towards the free components and thus achieve a dissociation.

Two recent studies of the human papillomavirus E7 protein, directed at the role of E7-specific sequences in the interaction with Rb and the E2F-Rb complex, reached conclusions similar to ours concerning the interaction of the E7 protein and E2F with distinct sites on Rb (20, 48). However, although an E7 CR2 peptide could block E7-mediated dissociation, a distinct functional equivalent of the E1A CR1 domain, which could block E2F-Rb interaction, could not be identified in E7. Rather, this inhibition required the full-length or near-full-length E7 protein. Although it is possible that E1A and E7 differ with respect to the mechanism outlined in Fig. 7, it is also possible that the E7 interaction that competes for E2F binding, equivalent to the E1A CR1 domain, is considerably weaker than that of E1A and requires stabilization by the second site of interaction.

On the basis of the data and the model shown in Fig. 7, we conclude that E1A makes two distinct contacts with the Rb protein as well as the p107 protein. This suggestion is consistent with other experiments that have shown independent interactions of E1A CR1 and CR2 sequences with the Rb protein (11). Clearly, the sequences recognized by the CR2 domain must not be involved in the interaction with the E2F transcription factor, on the basis of the formation of a trimeric complex. Sequences within the CR2 domain that are involved in the Rb interaction include the L-X-C-X-E motif that is shared in SV40 T antigen and the human papillomavirus E7 protein (14). This sequence is not found in E2F1 (18, 22, 42) or in the recently isolated DP1, another clone encoding E2F-like activity (16). The absence of this motif is consistent with the fact that E2F1 and E1A CR2 interact with distinct sequences of Rb.

Interestingly, this L-X-C-X-E motif is also found in several other cellular proteins identified as Rb-binding proteins (8), raising the possibility that one or more of these proteins could associate with the E2F-Rb complex and function in a manner similar to that of E1A. Indeed, the D-type cyclin polypeptides, which have recently been shown to interact with the cdk4 kinase and mediate the phosphorylation of Rb (24), contain this L-X-C-X-E motif (31, 49). Moreover, the cyclin D-cdk4-mediated phosphorylation of Rb apparently inhibits the ability of Rb to interact with E2F. The fact that E1A can interact with the E2F-Rb complex, dependent on the CR2 domain that contains the L-X-C-X-E motif, and that the D-type cyclins possess this same motif raises the possibility that a cyclin D-cdk4 complex could associate with the E2F-Rb complex in a manner similar to that of the association of E1A with the E2F-Rb complex. If a site for Rb phosphorylation that blocked the binding of Rb to E2F were to reside within the sequences that were recognized by the E1A CR1 domain, then this interaction and phosphorylation would be functionally analogous to the model as presented in Fig. 7.

REFERENCES

1. **Bagchi, S., P. Raychaudhuri, and J. R. Nevins.** 1990. Adenovirus E1A proteins can dissociate heteromeric cellular complexes involving the E2F transcription factor: a novel mechanism for E1A trans-activation. Cell 62:659–669.
2. **Bandara, L. R., and N. B. La Thangue.** 1991. Adenovirus E1A prevents the retinoblastoma gene product from complexing with a cellular transcription factor. Nature (London) 351:494–497.
3. **Blake, M. C., and J. C. Azizkhan.** 1989. Transcription factor E2F is required for efficient expression of the hamster dihydrofolate reductase gene in vitro and in vivo. Mol. Cell. Biol. 9:4994–5002.
4. **Cao, L., B. Faha, M. Dembski, L. H. Tsai, E. Harlow, and N. Dyson.** 1992. Independent binding of the retinoblastoma protein and p107 to the transcription factor E2F. Nature (London) 355:176–179.
5. **Chellappan, S., V. B. Kraus, B. Kroger, K. Munger, P. M. Howley, W. C. Phelps, and J. R. Nevins.** 1992. Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc. Natl. Acad. Sci. USA 89:4549–4553.
6. **Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins.** 1991. The E2F transcription factor is a cellular target for the Rb protein. Cell 65:1053–1061.
7. **Dalton, S.** 1992. Cell cycle regulation of the human cdc2 gene. EMBO J. 11:1797–1804.
8. **Defeo-Jones, D., P. S. Huang, R. E. Jones, K. M. Haskell, G. A. Vuocolo, M. G. Hanobik, H. E. Huber, and A. Oliff.** 1991. Cloning of cDNAs for cellular proteins that bind to the retinoblastoma gene product. Nature (London) 352:251–254.
9. **Devoto, S. H., M. Mudryj, J. Pines, T. Hunter, and J. R. Nevins.** 1992. A cyclin A-cdc2 kinase complex possesses sequence-specific DNA binding activity: p33 cdk2 is a component of the E2F-cyclin A complex. Cell 68:167–176.
10. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract isolated from mammalian nuclei. Nucleic Acids Res. 11:1475–1489.
11. **Dyson, N., P. Guida, C. McCall, and E. Harlow.** 1992. Adenovirus E1A makes two distinct contacts with the retinoblastoma protein. J. Virol. 66:4606–4611.
12. **Dyson, N., P. Guida, K. Munger, and E. Harlow.** 1992. Homologous sequences in adenovirus E1A and human papillomavirus

E7 proteins mediate interaction with the same set of cellular proteins. J. Virol. 66:6893–6902.
13. **Ewen, M. E., B. Faha, E. Harlow, and D. M. Livingston.** 1992. Interaction of p107 with cyclin A independent of complex formation with viral oncoproteins. Science 255:85–90.
14. **Figge, J., T. Webster, T. F. Smith, and E. Paucha.** 1988. Prediction of similar transforming regions in simian virus 40 large T, adenovirus E1A, and *myc* oncoproteins. J. Virol. 62:1814–1818.
15. **Giordano, A., J. H. Lee, J. A. Scheppler, C. Herrmann, E. Harlow, U. Deuschle, D. Beach, and B. R. Franza.** 1991. Cell cycle regulation of histone H1 kinase activity associated with the adenoviral protein E1A. Science 253:1271–1275.
16. **Girling, R., J. F. Partridge, L. R. Bandara, N. Burden, N. F. Totty, J. J. Hsuan, and N. B. LaThangue.** 1993. A new component of the transcription factor DRTF1/E2F. Nature (London) 352:83–87.
17. **Hamel, P. A., R. M. Gill, R. A. Phillips, and B. L. Gallie.** 1992. Transcriptional repression of the E2-containing promoters EIIaE, *c-myc*, and *RB1* by the product of the *RB1* gene. Mol. Cell. Biol. 12:3431–3438.
18. **Helin, K., J. A. Lees, M. Vidal, N. Dyson, E. Harlow, and A. Fattaey.** 1992. A cDNA encoding a pRB-binding protein with properties of the transcription factor E2F. Cell 70:337–350.
19. **Hiebert, S. W., S. P. Chellappan, J. M. Horowitz, and J. R. Nevins.** 1992. The interaction of RB with E2F coincides with an inhibition of the transcriptional activity of E2F. Genes Dev. 6:177–185.
20. **Huang, P. S., D. R. Patrick, G. Edwards, P. J. Goodhart, H. E. Huber, L. Miles, V. M. Garsky, A. Oliff, and D. C. Heimbrook.** 1993. Protein domains governing interactions between E2F, the retinoblastoma gene product, and human papillomavirus type 16 E7 protein. Mol. Cell. Biol. 13:953–960.
21. **Jelsma, T. N., J. A. Howe, J. S. Mymryk, C. M. Evelegh, N. F. A. Cunniff, and S. T. Bayley.** 1989. Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer, and induction of proliferating cell nuclear antigen. Virology 170:120–130.
22. **Kaelin, W. G., W. Krek, W. R. Sellers, J. A. DeCaprio, F. Ajchenbaum, C. S. Fuchs, T. Chittenden, Y. Li, P. J. Farnham, M. A. Blanar, D. M. Livingston, and E. K. Flemington.** 1992. Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties. Cell 70:351–364.
23. **Kaelin, W. G., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston.** 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. Cell 64:521–532.
24. **Kato, J. Y., H. Matsushime, S. W. Hiebert, M. E. Ewen, and C. J. Sherr.** 1993. Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. 7:331–342.
25. **Kimelman, D., J. S. Miller, D. Porter, and B. E. Roberts.** 1985. E1a regions of the human adenovirus and the highly oncogenic simian adenovirus 7 are closely related. J. Virol. 53:399–409.
26. **Kraus, V. B., J. A. Inostroza, D. Reinberg, and J. R. Nevins.** Interaction of the Dr1 inhibitory factor with TBP is disrupted by adenovirus E1A protein—a role in transcription activation and oncogenesis. Submitted for publication.
27. **Kraus, V. B., and J. R. Nevins.** Unpublished data.
28. **Lees, E., B. Faha, V. Dulic, S. I. Reed, and E. Harlow.** 1992. Cyclin E/cdk2 and cyclin A/cdk2 kinases associate with p107 and E2F in a temporally distinct manner. Genes Dev. 6:1874–1885.
29. **Lillie, J. W., M. Green, and M. R. Green.** 1986. An adenovirus E1a protein region required for transformation and transcriptional repression. Cell 46:1043–1051.
30. **Loeken, M. R., and J. Brady.** 1989. The adenovirus EIIA enhancer. Analysis of regulatory sequences and changes in binding activity of ATF and EIIF following adenovirus infection. J. Biol. Chem. 264:6572–6579.
31. **Matsushime, H., M. F. Roussel, R. A. Ashmun, and C. J. Sherr.** 1991. Colony stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. Cell 65:701–713.
32. **Means, A. L., J. E. Slansky, S. L. McMahon, M. W. Knuth, and P. J. Farnham.** 1992. The HIP1 binding site is required for growth regulation of the dihydrofolate reductase gene promoter. Mol. Cell. Biol. 12:1054–1063.
33. **Moran, E., B. Zerler, T. M. Harrison, and M. B. Mathews.** 1986. Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. Mol. Cell. Biol. 6:3470–3480.
34. **Mudryj, M., S. H. Devoto, S. W. Hiebert, T. Hunter, J. Pines, and J. R. Nevins.** 1991. Cell cycle regulation of the E2F transcription factor involves an interaction with cyclin A. Cell 65:1243–1253.
35. **Nevins, J. R.** 1992. E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. Science 258:424–429.
36. **Pines, J., and T. Hunter.** 1990. Human cyclin A is adenovirus E1A-associated protein p60 and behaves differently from cyclin B. Nature (London) 346:760–763.
37. **Pines, J., and T. Hunter.** 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J. Cell Biol. 115:1–17.
38. **Qian, Y., C. Luckey, L. Horton, M. Esser, and D. J. Templeton.** 1992. Biological function of the retinoblastoma protein requires distinct domains for hyperphosphorylation and transcription factor binding. Mol. Cell. Biol. 12:5363–5372.
39. **Qin, X. Q., T. Chittenden, D. M. Livingston, and W. G. Kaelin.** 1992. Identification of a growth suppression domain within the retinoblastoma gene product. Genes Dev. 6:953–964.
40. **Raychaudhuri, P., S. Bagchi, S. H. Devoto, V. B. Kraus, E. Moran, and J. R. Nevins.** 1991. Domains of the adenovirus E1A protein required for oncogenic activity are also required for dissociation of E2F transcription factor complexes. Genes Dev. 5:1200–1211.
41. **Schwarz, J., S. H. Devoto, E. J. Smith, S. Chellappan, L. Jakoi, and J. R. Nevins.** 1993. Interactions of the p107 and Rb proteins with E2F during the cell proliferative response. EMBO J. 12:1013–1020.
42. **Shan, B., X. Zhu, P.-L. Chen, T. Durfee, Y. Yang, D. Sharp, and W.-H. Lee.** 1992. Molecular cloning of cellular genes encoding retinoblastoma-associated proteins: identification of a gene with properties of the transcription factor E2F. Mol. Cell. Biol. 12:5620–5631.
43. **Shirodkar, S., M. Ewen, J. A. DeCaprio, J. Morgan, D. M. Livingston, and T. Chittenden.** 1992. The transcription factor E2F interacts with the retinoblastoma product and a p107-cyclin A complex in a cell cycle-regulated manner. Cell 68:157–166.
44. **Slansky, J. E., Y. Li, W. G. Kaelin, and P. J. Farnham.** 1993. A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter. Mol. Cell. Biol. 13:1610–1618.
45. **Weintraub, S. J., C. A. Prater, and D. C. Dean.** 1992. Retinoblastoma protein switches the E2F site from positive to negative element. Nature (London) 358:259–261.
46. **Whyte, P., H. E. Ruley, and E. Harlow.** 1988. Two regions of the adenovirus early region 1A proteins are required for transformation. J. Virol. 62:257–265.
47. **Whyte, P., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell 56:67–75.
48. **Wu, E. W., K. E. Clemens, D. V. Heck, and K. Munger.** 1993. The human papillomavirus E7 oncoprotein and the cellular transcription factor E2F bind to separate sites on the retinoblastoma tumor suppressor protein. J. Virol. 67:2402–2407.
49. **Xiong, Y., T. Connolly, B. Futcher, and D. Beach.** 1991. Human D-type cyclin. Cell 65:691–699.
50. **Zamanian, M., and N. B. La Thangue.** 1992. Adenovirus E1A prevents the retinoblastoma gene product from repressing the activity of a cellular transcription factor. EMBO J. 11:2603–2610.
51. **Zerler, B., B. Moran, K. Maruyama, J. Moomaw, T. Grodzicker, and H. E. Ruley.** 1986. Adenovirus E1A coding sequences that enable *ras* and *pmt* oncogenes to transform cultured primary cells. Mol. Cell. Biol. 6:887–899.